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Clinicopathologic Correlates of Loss of Heterozygosity in Wilms' Tumor: A Preliminary Analysis

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Wilms' tumor-specific loss of heterozygosity (LOH) for DNA markers located at chromosomes 11p13, 11p15, 16q, and 1p has been reported to occur in a minority of Wilms' tumors. We hypothesized that tumors classified by region of LOH would exhibit specific clinicopathologic patterns. We have therefore determined the constitutional and tumor genotypes for markers at 11p13, 11p15, 16q, and 1p in a large series of Wilms' tumor patients who were registered on a Pediatric Oncology Group study and on the National Wilms' Tumor Study, to determine whether tumor-specific LOH for any of these regions was associated with any specific phenotype.

Of 286 cases, 27% had LOH at both 11p13 and p15 (BOTH), 3% at 11p13 only, 8% at 11p15 only, and 62% at neither. Significant associations were found between younger age at diagnosis and LOH for BOTH, but not for 11p15 only, and between the presence of intralobar nephrogenic rests and LOH for BOTH. The incidence of nephrogenic rests (all types combined) and of bilateral tumors was the same in tumors with or without LOH. There was a negative association between anaplastic histology and LOH for 11p. There was no association between LOH on 11p and outcome as assessed by relapse-free and overall survival.

The associations between age at diagnosis and LOH are interpreted as suggesting the existence of a Wilms' tumor locus on 11p in addition to *WT1* at 11p13 and the putative *WT2* at 11p15. LOH for chromosome 16q was identified in 17% of 204 tumors and was associated with a significantly worse outcome. Outcome for patients with LOH for 1p was also worse but not significantly so.

Key words: loss of heterozygosity, nephrogenic rests, *WT1* and *WT2*

INTRODUCTION

Wilms' tumor-specific loss of heterozygosity (LOH) for DNA markers located at chromosomes 11p13 [1], 11p15 [2], 16q [3], and 1p [4] has been reported to occur in a minority of Wilms' tumors. The minimal region of overlap of LOH in a group of tumors can be used to infer the location of the underlying tumor gene. Wilms' tumors have been described with LOH restricted to chromosome 11p13 [1], the site of the Wilms' tumor suppressor gene, *WT1;* to 11p15 [1,2,5,6], the site of the putative *WT2;* and in some cases including both regions. In the 60–70% of tumors which do not harbor LOH for chromosome 11p, the second event might involve a genetic or epigenetic [7] alteration undetectable by LOH, or the mechanism of tumorigenesis in some cases might not involve loci on chromosome 11p. In those tumors with LOH at 11p, however, the specific region involved, 11p13 vs. 11p15, should imply the involvement of *WT1* vs. *WT2*.

We hypothesized that tumors classified by region of LOH would exhibit specific clinicopathologic patterns. In support of this hypothesis, the pattern of nephrogenic rests differs in patients with the 11p13 deletion-associated WAGR syndrome (*W*ilms' tumor, *A*niridia, *G*enitourinary malformations, mental *R*etardation) compared with the 11p15-linked Beckwith-Wiedemann syndrome [8]. Similar analyses using molecular markers to define the loci in nonsyndrome-associated cases have not been reported.

Although there is no evidence that the putative Wilms' tumor genes on chromosomes 16q and 1p play a role in the predisposition to, or early development of, Wilms' tumor, the occurrence of LOH for these regions might be expected to correlate with clinical features such as stage, histology, or prognosis if the LOH in fact pointed to the involvement of an underlying tumor gene.

We have therefore determined the constitutional and tumor genotypes for markers at 11p13, 11p15, 16q, and 1p in a large series of patients collected through the Pediatric Oncology Group to determine whether tumor-specific LOH for any of these regions was associated with any specific phenotype.

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MATERIALS AND METHODS

Subjects

Patients were accrued through the Pediatric Oncology Group Study 9046, "A Molecular Genetic Analysis of Wilms Tumor." This is a prospective study which includes patients younger than age 17 years with any renal tumor. Although not a requirement of this biological study, over 95% of the patients were independently registered on the third or fourth National Wilms' Tumor Study, thus making central pathology review and follow-up data available. Importantly, all patients were treated on uniform protocols defined by stage and histology [9]. Analyses of outcome included only patients with intrarenal Wilms' tumors of favorable or anaplastic histology [10] for whom both date of diagnosis and at least one follow-up report had been received.

DNA Analysis

DNA was extracted from frozen peripheral blood and tumor tissue and Southern blotted using standard procedures as previously reported [4]. Some polymorphic loci were analyzed using the polymerase chain reaction (PCR), with separation of the products on acrylamide gels and detection by ethidium bromide staining.

For chromosome 11p13, all cases were examined at the *FSH* and D11S16 loci, both of which are slightly telomeric of *WT1*. If constitutionally homozygous and therefore noninformative at both loci, one or more of *WT1*, *CAT*, D11S914, or D11S907 were examined until the case was informative for at least one locus. Similarly at 11p15, all cases were examined at *HRAS1* and *INS*, and, if noninformative, also at *TH* or D11S922. For chromosome 16q, D16S7 (16q24.3) and *HP* (16q22-q23) were analyzed in all cases and if either was noninformative, also at CTRB (16q24.1) or D16S260 (16q22-23), respectively. For chromosome 1p only one locus, D1Z2, was analyzed. Each chromosomal region, 11p13, 11p15, 16q, and 1p, was classified as LOH if a loss was seen at any locus; as retained, if at least one locus was informative; and noninformative if all loci were so.

Patients with bilateral tumors, when both were analyzed, were coded as LOH if either tumor displayed loss of the chromosomal segment.

Statistical Analysis

Age distributions were analyzed by the Wilcoxon two-sample test results, and stage, histology, and nephrogenic rest distributions by chi-square analysis. The two endpoints for outcome were relapse/progression-free survival (RFS) and overall survival (OS). Patients with persistent disease at last follow-up were not coded as failures unless they had evidence of progressive disease from time of initial treatment. One patient who died of toxicity without prior relapse was treated as censored at time of death in the RFS analysis. Relapse-free and OS curves were compared using the log-rank test [11]. Relative risks were calculated using the Cox proportional hazards model, with and without adjustment for stage or anaplastic histology [12].

TABLE I. Incidence of LOH on Chromosome 11p by Region

	No. of patients (%)
LOH p13 only	10 (3.4)
LOH p15 only	22 (7.4)
LOH both	76 (25.7)
LOH neither	178 (60.1)
Not informative[a]	10 (3.4)
Total	296 (100)

[a]Cases not informative for one region but which retained heterozygosity at the other. Cases which had LOH for one region but were noninformative for the other were included in the appropriate LOH category.

TABLE II. Age at Diagnosis by Region of LOH on 11p

	No.	Mean age (months)
LOH p13 only	10	28.1
LOH p15 only	22	42.8
LOH both	76	33.1
LOH neither	178	45.6
NI either	10	
	296	
p13 (± p15)[a]		
Loss	86	32.5
Retain	198	45.6 $P = 0.0002$
p15 (± p13)[a]		
Loss	98	35.2
Retain	195	44.0 $P = 0.011$

[a]Excluding patients whose tumors were noninformative for p13 (± p15), 12 pts; and p15 (± p13), 3 pts.

RESULTS

Chromosome 11p

The distribution of cases classified by region of LOH on chromosome 11p is shown in Table I. In addition to mutually exclusive classification as loss of 11p13 only, 11p15 only, both (BOTH), or neither, cases can also be classified considering each region independently, i.e., 86 cases with loss at p13 including 10 at 11p13 only and 76 at BOTH, and 98 cases with loss at p15 including 22 at 11p15 only and 76 at BOTH. Obviously the latter groups overlap substantially but due to the larger numbers were more amenable to statistical analysis.

The average age at diagnosis for the subgroups is shown in Table II. Ages were significantly younger for those with LOH at 11p13 (BOTH and p13 only) and with LOH at 11p15 (BOTH and p15 only) relative to those with no loss. While the groups with LOH p13 only or p15 only were too small for statistical analysis, there was an obvious trend to younger age for those with LOH at p13 only (28.1 months) but not for those with LOH 11p15 only (42.8 months).

TABLE III. Nephrogenic Rests by LOH 11p

	No.[a] evaluable	ILNR only (%)	PLNR only (%)	Both PLNR + ILNR (%)	None (%)
LOH p13 only	6	33	0	17	50
LOH p15 only	16	25	19	6	50
LOH both	58	28	16	2	53
LOH neither	127	13	29	5	54
NI either	8				
All	215	18	24	5	53
		PLNR ± ILNR		ILNR ± PLNR	
p13 (± p15)[b]					
Loss	64	12		21	
Retain	141	46		27	
		OR = 0.48; $P = 0.046$		OR = 2.1; $P = 0.049$	
p15 (± p13)[b]					
Loss	74	15		23	
Retain	138	44		26	
		OR = 0.54; $P = 0.079$		OR = 1.94; $P = 0.06$	

[a]Excluding cases where no slides were submitted (19 patients) or where the presence of nephrogenic rests was indeterminable (62 patients).
[b]Cases were excluded if noninformative for the region indicated; p13 ± p15 (10 patients); p15 ± p13 (3 patients).

Two hundred seven patients were evaluable for the presence of nephrogenic rests in the nephrectomy specimen. Nephrogenic rests are of two types: intralobar and perilobar abbreviated as ILNR and PLNR, respectively. A comparison of the incidence and type of nephrogenic rest by region of LOH on chromosome 11p is shown in Table III. Although rests of any type were present in approximately 50% of each group with or without LOH, a significantly positive association was observed between LOH p13 and intralobar rests (± perilobar rests) (OR = 2.1) and a significantly negative association with perilobar rests (± intralobar rests) (0.48). The same trends were observed at p15 but these did not reach statistical significance.

Tumors were classified into favorable or anaplastic histology using National Wilms' Tumor Study (NWTS) criteria [10]. As shown in Table IV, anaplastic histology was significantly less common in tumors with LOH p13 (BOTH and p13 only) and p15 (BOTH and p15 only) relative to those without LOH. The same trend was observed in the small p13 only group but not when considering LOH p15 only.

There was no evidence for association between LOH and tumor surgical stage I-IV, or with the incidence of bilateral tumors (data not shown).

There were too few cases with anomalies to analyze statistically. Interestingly, three of eight cases with Beckwith-Wiedemann syndrome had LOH at 11p15 and one had LOH restricted to 11p13. Cryptorchidism occurred in two cases with LOH at 11p15 only.

There was no difference in outcome, either in RFS or OS for children with tumors with LOH at 11p13 and/or 11p15 compared with those whose tumors retained heterozygosity (data not shown).

TABLE IV. Histological Classification by LOH 11p*

	Favorable	Anaplastic
LOH p13 only	9	0 (0%)
LOH p15 only	19	2 (11%)
LOH both	70	2 (3%)
LOH neither	132	21 (16%)
NI either	7	1
Total	237	26
p13 (± p15)[a]		
Loss	79	2 (3%)
Retain	149	23 (15%)
		OR = 0.16; $P = 0.006$
p15 (± p13)[b]		
Loss	89	4 (5%)
Retain	145	22 (15%)
		OR = 0.30; $P = 0.029$

*Including only cases with favorable or anaplastic (focal or diffuse) histology. Other histological patterns were too infrequent to allow meaningful comparison.
[a]Excluding cases which were noninformative for the respective region; p13 (9 patients), p15 (3 patients).

Chromosomes 16q and 1p

LOH for chromosome 16q occurred in 17% of 204 informative cases and for chromosome 1p in 12% of 175 informative cases with follow-up information available. No association between LOH at 16q or 1p and either stage or histology was observed. LOH at 16q was, however, associated with a significantly lower RFS and OS. Outcome postrelapse appeared particularly poor for the seven

patients with LOH at 16q at initial diagnosis, of whom only one survived, compared with 9 of 12 relapsed patients without LOH. There were 4 relapses among the 21 patients with LOH at 1p vs. 12 of 154 patients without LOH, but this difference did not reach statistical significance.

DISCUSSION

Since tumor-specific LOH is ascertained by comparison with a presumed normal tissue, usually either peripheral lymphocytes or normal kidney, this genetic event is, by definition, a somatic occurrence. LOH has been thought to represent the second hit affecting a tumor-suppressor gene, i.e., loss of the remaining normal allele following mutation or deletion of the first allele [13]. While the initial mutational event may be either constitutional or somatic, there is no a priori reason why tumors with LOH should more frequently harbor a constitutional rather than a somatic initial event. Thus, although different regions of LOH infer the involvement of specific genes, and might therefore be expected to correlate with specific features of the tumor such as stage or histological subtype, one would not expect the presence of LOH to correlate with indicators of the timing of the first event, i.e., prezygotic vs. somatic.

The association between LOH at 11p and age at diagnosis is therefore surprising. It is possible that younger age at diagnosis is determined by the biological effect of loss of the involved gene rather than indicating the occurrence of a constitutional genetic event. This possibility is supported by the findings that bilateral tumors and nephrogenic rests (all types combined), presumed hallmarks of constitutional or very early somatic mutations, were no more frequent in cases with LOH.

Younger age at diagnosis was observed with tumors with LOH at 11p13 (p13 $\pm$ p15) and at 11p15 (p15 $\pm$ p13) compared with those without LOH, but these groups overlapped substantially. A definite trend to younger age was observed in cases with LOH at 11p13 only, but not with LOH at 11p15 only, although the groups were too small to be significantly different. These data would suggest that loss of function of a gene at 11p13 accounts for the age difference.

The Wilms tumor gene, *WT1,* is located at 11p13. At least two large studies, however, have shown that less than 10% of Wilms' tumors harbor a *WT1* mutation [14,15], considerably less than the 30% incidence of tumor-specific LOH found in this and other studies [16,17]. It is, therefore, difficult to attribute the difference in age at diagnosis to involvement of *WT1.* Similarly, although LOH, as a single genetic event, could affect more than one locus simultaneously (i.e., at p13 and p15), involvement of *WT1* remains somewhat implausible because of the infrequency of mutation.

Likewise, although LOH may alter the imprinted tumor-associated loci at 11p15 as a single event, there was no suggestion of a younger average age at diagnosis in the 22 cases with LOH at 11p15 only. It is therefore also difficult to invoke involvement of these loci in the age differential in cases with loss at both 11p13 and 11p15.

We would therefore propose that these findings support the existence of a Wilms' tumor locus on chromosome 11p, in addition to *WT1* and to those suspected to be located at 11p15. Given that 30% of Wilms' tumors have LOH which extends to 11p13, the putative locus may be located at 11p13. Given the limited numbers of cases with LOH at 11p13 only or at 11p15 only, these results must be considered preliminary. Additional cases are being analyzed for their 11p genotype and the cases with LOH at 11p13 only are being assayed for *WT1* mutations. These additional data should serve to strengthen the statistical analyses and to more specifically address the role of *WT1* involvement. Since no associations were found between the stage of the tumor and loss of any region, this clinical feature is likely determined by other factors apart from the specific locus underlying the etiology of the tumor.

It might have been assumed, based on associations between the WAGR syndrome and intralobar rests, and between Beckwith-Wiedemann syndrome and perilobar rests [8], that the type of rest was determined by differential involvement of loci at 11p13 or 11p15. Interestingly, we found a positive association between intralobar rests and LOH for both regions. This might indirectly reflect the earlier development of tumors with LOH, since intralobar rests represent an earlier stage of nephrogenesis, or be related to the fact that LOH potentially involves more than one genetic locus at the same time.

LOH 11p appears to be less frequent in tumors with anaplastic histology. Others have shown that *p53* mutation may underlie the development of anaplasia [18,19] but it is also possible, based on our results, that the etiology of anaplastic tumors does not involve loci on 11p and that anaplasia does not simply represent progression from favorable histology.

Loci on chromosomes 16q and 1p have not been implicated in the etiology of Wilms' tumor and our data do not support associations between LOH for these regions and any features of the tumor except outcome. It is most likely, therefore, that these loci are involved in the progression of these tumors to a more resistant phenotype. Confirmation of the prognostic importance of LOH 16q and possibly 1p, in addition to the known clinical prognostic factors of stage and histological classification, may allow further refinement of an individual's therapy for Wilms' tumor.

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COMMENTARY

That LOH occurs at several loci (including 11p13, 11p15, 16q, and 1p) in a minority of Wilms' tumors has been well established. Thus far, most investigators have used LOH data to discover the location of tumor-suppressor genes. Dr. Paul Grundy's laboratory, however, has taken a different avenue. Using data from the NWTS group, they have been able to associate LOH of different regions with specific clinicopathologic patterns (e.g., LOH at 16q and poor outcome). In the current manuscript, based on the associations between age at diagnosis and LOH, Grundy et al. postulate the existence of a third Wilms' tumor locus on 11p (in addition to *WT1* at 11p13 and the putative Wilms' tumor gene *WT2* at 11p15). The authors indicate that the third supposed locus may be located at 11p13. This proposal is quite intriguing and might explain, at least in part, the somewhat disappointing incidence of *WT1* mutations in Wilms' tumor, despite the relatively high incidence of LOH (~40%) in the region containing the *WT1* gene. Could *WIT-1* (see manuscript by Hewitt et al. in this issue, page 456) be this third locus?